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## LIVER DISEASE

## Liver repopulation with bone marrow derived cells improves the metabolic disorder in the Gunn rat

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**Background:** Reversible ischaemia/reperfusion (I/R) liver injury has been used to induce engraftment and hepatic parenchymal differentiation of exogenous  $\beta_2$ -microglobulin<sup>−</sup>/Thy1<sup>+</sup> bone marrow derived cells.

**Aim:** To test the ability of this method of hepatic parenchymal repopulation, theoretically applicable to clinical practice, to correct the metabolic disorder in a rat model of congenital hyperbilirubinaemia.

**Methods and results:** Analysis by confocal laser microscopy of fluorescence labelled cells and by immunohistochemistry for  $\beta_2$ -microglobulin, 72 hours after intraportal delivery, showed engraftment of infused cells in liver parenchyma of rats with I/R, but not in control animals with non-injured liver. Transplantation of bone marrow derived cells obtained from GFP-transgenic rats into Lewis rats resulted in the presence of up to 20% of GFP positive hepatocytes in I/R liver lobes after one month. The repopulation rate was proportional to the number of transplanted cells. Infusion of GFP negative bone marrow derived cells into GFP positive transgenic rats resulted in the appearance of GFP negative hepatocytes, suggesting that the main mechanism underlying parenchymal repopulation was differentiation rather than cell fusion. Transplantation of wild type bone marrow derived cells into hyperbilirubinaemic Gunn rats with deficient bilirubin conjugation after I/R damage resulted in 30% decrease in serum bilirubin, the appearance of bilirubin conjugates in bile, and the expression of normal UDP-glucuronyltransferase enzyme evaluated by polymerase chain reaction.

**Conclusions:** I/R injury induced hepatic parenchymal engraftment and differentiation into hepatocyte-like cells of bone marrow derived cells. Transplantation of bone marrow derived cells from non-affected animals resulted in the partial correction of hyperbilirubinaemia in the Gunn rat.

The pioneering work of Lagasse *et al*<sup>1</sup> on the mouse with fumarylacetoacetate hydrolase (FAH <sup>−/−</sup>) deficiency, an animal model of tyrosinaemia type I, has shown that bone marrow transplantation with purified haematopoietic stem cells from wild type animals could repopulate the liver and cure the inherited metabolic disorder. Enthusiasm for this therapeutic approach has been tempered by the finding that cell fusion, rather than true differentiation, was responsible for liver repopulation of cells with normal enzyme activity.<sup>2</sup> Moreover, the metabolic defect in the FAH <sup>−/−</sup> mouse leads to massive hepatocyte death, possibly creating the necessary conditions for extensive liver repopulation with exogenous cells. However, in most inherited disorders of liver metabolism the metabolic defect does not lead to liver parenchymal cell loss, and several investigators have demonstrated that in such conditions repopulation with exogenous cells is insignificant.<sup>3</sup>

Interruption of blood flow to an organ or tissue (ischaemia) and subsequent reperfusion leads to an acute inflammatory response that may cause significant cellular damage. Ischaemia/reperfusion (I/R) is a powerful stimulus for stem cell homing and repair of the injured tissue, as demonstrated in experimental cell therapy of myocardial infarction.<sup>4</sup> Reversible I/R injury of the liver has been documented in various clinical settings, including partial liver resection (Pringle manoeuvre), liver transplantation, and haemorrhagic shock with fluid resuscitation. Hepatocytes are sensitive to hypoxic damage and once injured about half the cells will undergo necrosis or apoptosis.<sup>5</sup>

The aim of this study was to verify whether reversible I/R injury of selected liver lobes can induce hepatic engraftment of bone marrow derived cells, leading to further differentiation into hepatocytes and to correction of an inherited metabolic disorder. It is possible that such a model of reversible partial

liver injury could be applied in clinical practice to repopulate the liver of patients with genetic liver disease with normal exogenous liver cells, in order to correct the metabolic disorder.

## METHODS

### Animals

All studies with animals were conducted under protocols approved by the Italian Ministry of Health, in accordance with Italian law (DL 116/92). Lewis rats were obtained from Charles River (Wilmington, Massachusetts, USA). Transgenic rats carrying enhanced green fluorescent protein (GFP) were a kind gift from Dr Masaru Okabe, Osaka, Japan. These animals express enhanced GFP as non-secreted cytoplasmic protein throughout the body except in peripheral erythrocytes and hair.<sup>6</sup> Jaundiced Gunn (JJ) rats and the non-hyperbilirubinaemic wild type (++, HSD:WT) were from Harlan, Indianapolis, Indiana, USA.

### Isolation of bone marrow derived cells

$\beta_2$ -Microglobulin ( $\beta_2m$ )<sup>−</sup>/(Thy-1)<sup>+</sup> cells (from now on referred to as bone marrow derived cells (BMDCs)) were isolated using a two step magnetic bead cell sorting method, as previously described.<sup>7</sup>

### Ischaemia/reperfusion injury

Laparotomy was carried out under pentobarbitone (pentobarbital) anaesthesia (60 mg/kg). The animal was given 10 IU heparin intravenously and the blood supply to the right lobe of

**Abbreviations:**  $\beta_2m$ ,  $\beta_2$ -microglobulin; BMDC, bone marrow derived cell; DAPI, 4',6'-diamidino-2-phenylindole; FAH, fumarylacetoacetate hydrolase; GFP, green fluorescent protein; I/R, ischaemia/reperfusion; JJ, jaundiced Gunn

the liver (corresponding to about 30% of total liver mass) was occluded with an atraumatic vascular clamp for 60 minutes. The body temperature was maintained at  $37 \pm 0.3^\circ\text{C}$  by a heating lamp. Reperfusion was initiated by removal of the clamp. Under these experimental conditions, a significant increase in necrotic and apoptotic hepatocytes is observed one hour after reperfusion.<sup>5</sup> The animals received 1 ml of sterile saline intraperitoneally, the wound was closed in layers with 4-0 silk and wound staples, and the rats were allowed to recover. Twenty four hours after reperfusion, the animals were re-anaesthetised, the abdomen reopened, and BMDCs suspended in 0.9% saline solution were seeded in the test animals through the portal vein into the right lobe of the liver. Control rats received saline solution alone.

### Evaluation of liver parenchymal repopulation

The degree of hepatic engraftment achieved was monitored by two different criteria: fluorescent staining of BMDCs with a membrane bound dye, and genetic labelling (GFP-transgenic animals). A description of the experimental groups is given in table 1.

### Fluorescent staining of BMDCs

BMDCs were stained with PKH26 red fluorescent cell linker (6  $\mu\text{mol/l}$ ). Three experimental groups of Lewis inbred male rats were studied: *group A* ( $n = 9$ ), animals with intraportal infusion of  $5 \times 10^6$  BMDCs in the absence of I/R injury; *group B* ( $n = 16$ ), animals with intraportal infusion of BMDCs following I/R injury; *group C* ( $n = 6$ ), animals with I/R injury without BMDCs infusion.

### GFP labelled cells

Two different experimental protocols were used:

#### First series (GFP positive cells into GFP negative animals)

BMDCs were isolated from GFP positive transgenic rats and transplanted into Lewis rats. This series included four experimental groups: *group D* ( $n = 5$ ), rats with I/R injury and transplantation of  $7 \times 10^6$  BMDCs, and control *group E* ( $n = 3$ ), rats with I/R injury without cell transplantation (both groups were studied 72 hours after cell infusion). *Group F* ( $n = 7$ ), rats with I/R injury and transplantation of different amounts of BMDCs ( $1 \times 10^6$ ,  $n = 2$ ;  $7 \times 10^6$ ,  $n = 3$ ; and  $10 \times 10^6$ ,  $n = 2$ , respectively) and control *group G* ( $n = 6$ ), rats with I/R injury without cell transplantation. In group F, two female rats received  $10 \times 10^6$  BMDCs isolated from male animals, in order to

use the Y chromosome as a second independent marker of transplanted cells. Both group F and group G were studied one month after cell infusion. All animals received daily immunosuppressive treatment with ciclosporine A, 15 mg/kg.

#### Second series (GFP negative cells into GFP positive animals)

To evaluate the occurrence of fusion,  $7 \times 10^6$  GFP negative BMDCs were infused into each GFP positive animal following I/R (*group H*,  $n = 6$ ), while controls received vehicle only (*group I*;  $n = 4$ ). In these conditions, differentiation of BMDCs should produce GFP negative hepatocytes, while fusion between haematopoietic cell lineages and liver parenchymal cells should result in GFP positive hepatocytes. The animals were studied one month after cell infusion. All animals received daily immunosuppressive treatment with ciclosporine A, 15 mg/kg.

### Collection of samples

The animals were killed by exsanguination under anaesthesia and liver samples were collected from the postischaemic and the non-ischaemic lobes. Samples were either fixed in phosphate buffered formalin for immunohistochemical analysis or frozen in dry ice and stored at  $-80^\circ\text{C}$  until fluorescent microscopy analysis.

### Histology, immunohistochemistry, and FISH

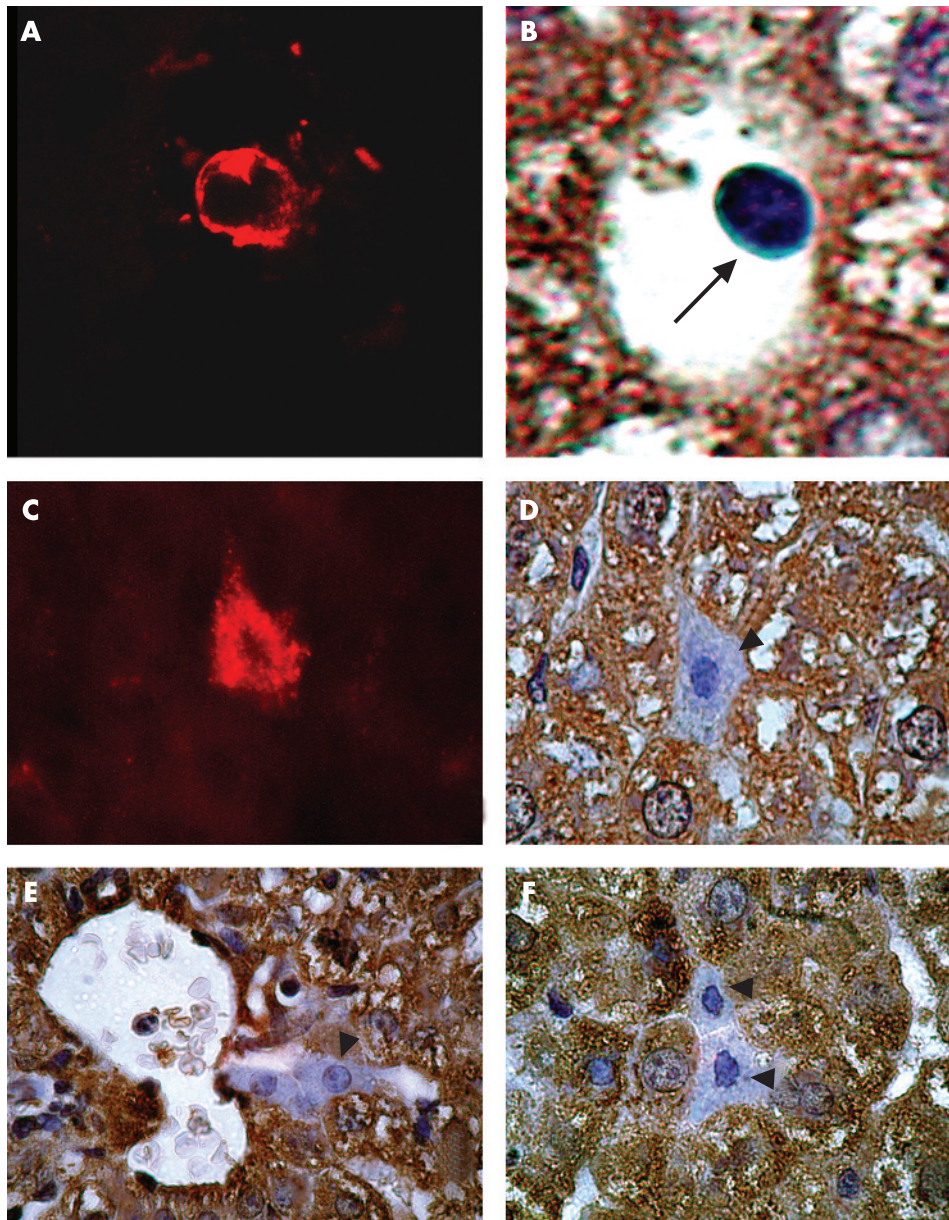
Liver tissue was fixed in 10% formalin and embedded in paraffin. Sections 5  $\mu\text{m}$  thick were cut and stained with haematoxylin-eosin for morphological analysis and for counting the fluorescent stained cells. Immunohistochemistry was undertaken with antibodies against  $\beta_2\text{m}$  (Santa Cruz, Santa Cruz, California, USA), GFP (Abcam, Cambridge, UK), or CK18 (Progen, Heidelberg, Germany). Fluorescent stained cells were analysed using confocal laser microscopy. At least 500 hepatocytes were counted per animal, and the number of fluorescent cells was expressed as per cent of counted hepatocytes. Tissue sections were deparaffinised, rehydrated, and either treated for 15–20 minutes at room temperature with 0.06% Triton X-100 and 0.1%  $\text{H}_2\text{O}_2$  for  $\beta_2\text{m}$  and GFP antibodies, or microwaved at 750 W for 10 minutes in 10 mM citrate buffer, pH 6.0, for CK18 antibody, and cooled down for at least 45 minutes. After washing in phosphate buffered saline (PBS), sections were incubated with normal serum in PBS for one hour at room temperature and for 30 minutes in an avidin/biotin blocking kit (Vector, Vinci-Biochem, Firenze, Italy). Sections were then incubated with the primary antibodies for two hours at  $37^\circ\text{C}$  ( $\beta_2\text{m}$  1:20; CK18 1:20) or for one hour at

**Table 1** Summary of experimental groups

Group	Procedure	Harvest	n
Cell tracking with fluorescent dye	Transplantation of fluorescent labelled BMDCs (PKH26)		
A	$5 \times 10^6$ BMDCs ip in the absence of I/R injury	72 h	9
B	$5 \times 10^6$ BMDCs ip after I/R injury	72 h	16
C	Vehicle only after I/R injury	72 h	6
Cell tracking with GFP and Y chromosome	Transplantation of GFP positive BMDCs		
D	$7 \times 10^6$ BMDCs after I/R injury	72 h	5
E	vehicle only after I/R injury	72 h	3
F	$1 \times 10^6$ , $7 \times 10^6$ , and $10 \times 10^6$ BMDCs after I/R injury	1 m	7
G	Vehicle only after I/R injury	1 m	6
Investigating cell fusion	Transplantation of BMDCs from wild type into GFP positive rats		
H	$7 \times 10^6$ BMDCs after I/R injury	1 m	6
I	Vehicle only after I/R injury	1 m	4
Assessing liver specific metabolic function in vivo	Transplantation of BMDCs from wild type into Gunn rats		
K	$10 \times 10^6$ BMDCs after I/R injury	1 m	6
L	Vehicle only after I/R injury	1 m	6

BMDC, bone marrow derived cell; GFP, green fluorescent protein; ip, intraportal; I/R, ischaemia/reperfusion.





**Figure 1** Identification of  $\beta_2$ -microglobulin ( $\beta_2$ m) negative cells in liver sections 72 hours after intraportal infusion of bone marrow derived cells (BMDCs). Liver sections from a control animal without ischaemia/reperfusion (I/R) injury (group A). (A) Typical appearance at confocal microscopy of a fluorescence labelled cell in non-injured liver parenchyma. (B) At immunohistochemistry for  $\beta_2$ m a small cell with ovoid nucleus, condensed chromatin, and a small rim of  $\beta_2$ m negative cytoplasm is visible inside a liver sinusoid (arrow). Liver sections from animals with I/R injury (group B). (C) Typical appearance at confocal microscopy of a fluorescence labelled cell in injured liver parenchyma. (D) (E) (F) At immunohistochemistry,  $\beta_2$ m negative cells with smaller nuclei than the surrounding hepatocytes are integrated within liver parenchyma; nuclear chromatin looks less condensed than in group A (arrowheads) and the cells acquire a polygonal shape with a large cytoplasm. (Original magnification: (A), (B)  $\times 100$ ; (C), (D), (E), (F)  $\times 40$ .)

room temperature (GFP 1:1200). The biotin conjugated secondary antibody was applied followed by incubation with peroxidase-streptavidin reagent, both for 30 minutes at room temperature (peroxidase vectastain elite ABC kit universal for GFP and CK18; peroxidase vectastain ABC kit, goat IgG, for  $\beta_2$ m (Vector, Vinci-Biochem)). The immunoreaction was visualised by developing sections with diaminobenzidine (Dako Cytomation, Milan, Italy). Controls consisted of incubation with normal serum instead of the primary antibody and of tissue sections expressing or not the specific antigens.

#### Dual staining protocol GFP/ $\beta_2$ m

The anti-GFP antibody was detected by biotin labelled swine anti-rabbit (1:400, Dako, Glostrup, Denmark) after avidin/biotin and serum blocking, followed by streptavidin-alkaline phosphatase (1:50, Dako) and visualisation with Alexa 488 (Invitrogen, Paisley, UK). The goat polyclonal anti- $\beta_2$ m antibody (1:20) was used after avidin/biotin and protein blocking followed by donkey anti-goat labelling with vector red (Vector Labs, Burlingame, California, USA). DAPI (4',6-diamidino-2-phenylindole) counterstaining was carried out before mounting.

#### Dual staining protocol, GFP/albumin

The polyclonal rabbit anti-albumin antibody (1:2000, Dako) was used, after the blocking steps, followed by biotin labelled swine anti-rabbit, streptavidin-alkaline phosphatase, and vector red development, as above. Following multiple washing steps and blocking with avidin/biotin and total protein block (Dako) the GFP antibody was applied (with PBS/normal goat serum) followed by goat anti-rabbit Alexa 488 (Invitrogen). DAPI counterstaining was carried out as above.

At least 500 hepatocytes were counted at high power field ( $200\times$ ), and the presence of BMDC derived hepatocyte-like cells (GFP positive or negative) was confirmed by examination at  $400\times$ . The number of BMDC derived hepatocyte-like cells was expressed as per cent of total counted hepatocytes.

The bone marrow origin of hepatocyte-like cells was confirmed by fluorescent in situ hybridisation (FISH) for the Y chromosome using a rat Y/12 chromosomal paint (Y-Cy3; 12-FITC, CA-1631, Cambio, Cambridge, UK) combined with immunohistochemistry for GFP. Sections were dewaxed and then microwaved for 10 minutes in sodium citrate (2.94 g/l). The primary antibody was an anti-GFP (1:500) (catalogue No

A11122, Molecular Probes, Eugene, Oregon, USA) and the secondary antibody was an Alexa Fluor 647-anti-rabbit (Molecular Probes). Sections were incubated in 1 M sodium thiocyanate for 10 minutes at 80°C, washed in PBS, and then digested in pepsin (0.4% wt/vol) in 0.1 M HCl, at 37°C for four minutes. After quenching in glycine (0.2% vol/vol), the sections were rinsed in PBS, post-fixed in paraformaldehyde (4% wt/vol), dehydrated through graded alcohols, and air dried. The chromosome paints were added to the sections, sealed under glass with rubber cement, heated to 60°C for 10 minutes, and incubated overnight at 37°C. The slides were rinsed in 0.5×SSC (NaCl/sodium citrate) and then in PBS and mounted in Vectashield (Vector Laboratories) for confocal microscopy.

### Correction of the metabolic disorder in the Gunn rat

Gunn j/j rats with congenital deficiency of bilirubin glucuronyltransferase were subjected to I/R injury. Forty eight hours after the procedure, the animals received an intraportal infusion of either  $10 \times 10^6$  BMDCs isolated from wild type syngeneic animals (group K,  $n = 6$ ) or saline solution (group L,  $n = 6$ ). Blood was collected from the tail vein before surgery and 30 days after cells infusion. After 30 days, the animals were anaesthetised and bile was collected on ice and in the dark. Alkaline methanolysis—HPLC analysis of bile pigments in bile and plasma—was carried out as described previously.<sup>8</sup> Liver tissue was obtained for RNA extraction as described below.

### RNA extraction and PCR analysis

Total RNA was extracted from snap frozen liver tissue using Tri Reagent solution (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed with Moloney murine leukaemia virus reverse transcriptase (2.5 U/ $\mu$ l) in a final volume of 20  $\mu$ l containing: buffer (500 mmol/l KCL, 100 mmol/l Tris-HCl, pH 8.3),  $MgCl_2$  (5 mmol/l), dNTPs (1 mmol/l), random examers (2.5  $\mu$ mol/l), and RNase inhibitor (1 U/ $\mu$ l). After an incubation period of 10 minutes at room temperature, the cDNA synthesis was carried out in a thermocycler (Perkin Elmer, Milan, Italy) for 30 minutes at 42°C.

Polymerase chain reaction (PCR) was carried out using the following specific primers that amplified a 600 base pair (bp) fragment of the sequence of rat UGT1A1, the only uridine diphosphate glucuronosyltransferase isoform that preferentially binds bilirubin: UGT1A1 *forward* 5'-GAA AGA TTA CCC CAG GCC CAT CAT GCC-3'; UGT1A1 *reverse* 5'-GCC AGG TCC AGA GGC TCG ATA GGA CG-3'. The specificity and efficiency of the PCR reaction was increased using the Eppendorf HotMasterMix (Eppendorf, Milan, Italy), according to the manufacturer's instructions. The cycle employed was: two minutes at 94°C, 38 cycles of one minute at 95°, 40 seconds at 58°C, and 40 seconds at 72°C, followed by seven minutes at 72°C, before ending at 4°C. PCR products were digested with *Mva*I (Roche Diagnostics, Monza, Milan, Italy) and analysed on 7% acrylamide gel, visualised by ethidium bromide and then silver stained.

### Statistical analysis

Analyses were done using the Mann–Whitney test (two tailed) and the correlation analysis in the GraphPad Prism software program (San Diego, California, USA).

## RESULTS

### PKH26 labelled BMDCs

More fluorescent cells were observed in livers of group B (rats that underwent I/R) than of sham operated group A rats (mean (SD): 0.28 (0.11) vs 0.04 (0.01) PKH26 labelled cells/100 hepatocytes;  $p < 0.0001$ ; fig 1). Immunohistochemistry for  $\beta_2m$  showed the presence of  $\beta_2m$  negative cells integrated in the

liver parenchyma of I/R rats and having morphological features of hepatocyte differentiation (group B, fig 1). A few  $\beta_2m$  negative cells were occasionally observed in sinusoidal spaces. In group A, as well as in non-ischaemic liver lobes of group B, small  $\beta_2m$  cells were sporadically observed only inside portal branches or sinusoids (fig 1). No  $\beta_2m$  negative cells were observed in the non-infused controls (group C).

### GFP labelled BMDCs

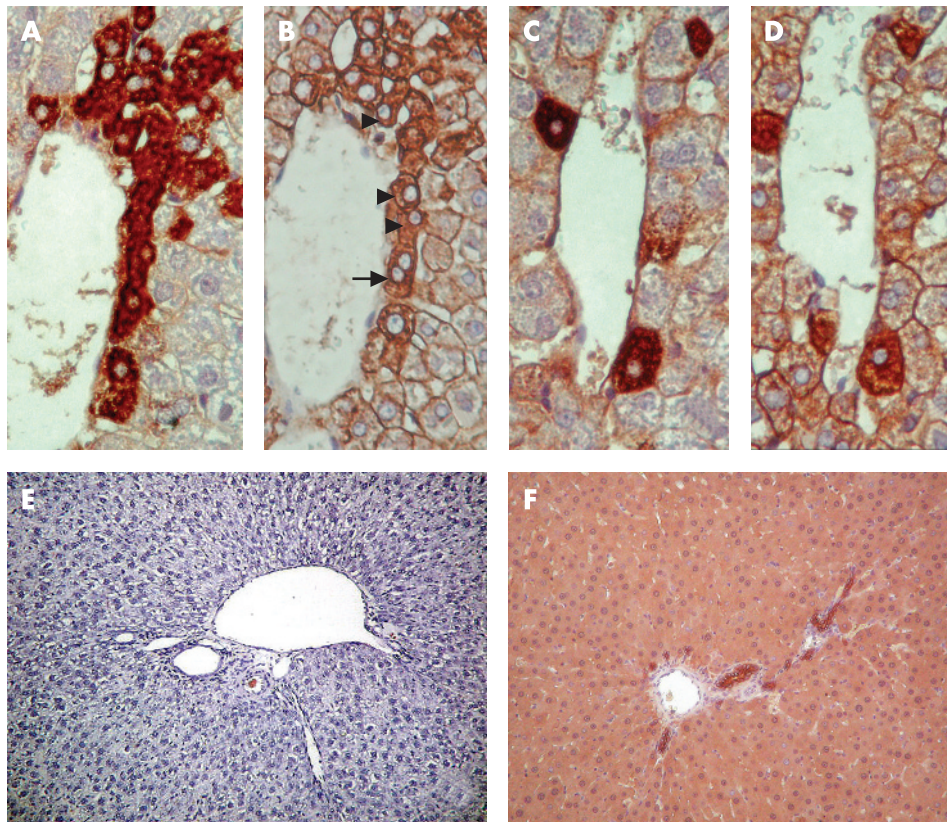
At immunohistochemistry, GFP was homogeneously expressed in liver parenchymal cells of transgenic animals. Seventy two hours after transplantation, GFP positive, CK18 positive hepatocyte-like cells were found in ischaemic lobes of Lewis rats with I/R at a frequency of 0.39 (0.31) ( $n = 5$ ), as per cent of total hepatocytes in the liver (group D; fig 2, panels A, B, C, D), while no GFP positive cells were present in controls without cell infusion (group E). The frequency of repopulation at 72 hours with GFP labelled hepatocytes was comparable to that observed with PKH26 labelled cells (group A, 0.28 (0.11); group D, 0.39 (0.31);  $p > 0.05$ ). Immunofluorescence analysis showed that after 72 hours some GFP positive cells stained negative for  $\beta_2m$  (fig 3D). The extent of repopulation with GFP positive hepatocyte-like cells was further evaluated one month after transplantation (group F), and was found to be proportional to the number of infused cells (linear regression:  $r^2 = 0.9117$ ; exponential growth:  $R^2 = 0.9575$ ; fig 4), up to approximately 20% of total liver parenchymal cells after infusion of  $10 \times 10^6$  BMDCs (fig 5, panels A to E). Immunofluorescence analysis showed that GFP positive hepatocyte-like (bone marrow derived) cells stained more weakly for albumin than the surrounding native hepatocytes (fig 6). GFP positive endothelial-like cells were also observed in the ischaemic lobes of Lewis rats one month after transplantation, while they were not found in non-ischaemic parenchyma (fig 5F). The use of a double marker (that is, FISH for Y chromosome and co-staining with anti-GFP antibody) further confirmed the bone marrow origin of the GFP positive liver parenchymal cells. Liver parenchyma of female rats receiving male BMDCs showed the presence of clusters of GFP positive hepatocytes incorporating the Y chromosome (fig 7). Again, no GFP positive cells were present in controls without cell infusion (group G). Infusion of BMDCs isolated from Lewis rats into GFP-transgenic rats was associated with the appearance of GFP negative, CK18 positive hepatocytes (group H, fig 8), while no GFP negative hepatocytes were observed in controls (group I). The proportion of repopulation of GFP negative hepatocytes in GFP positive livers infused with  $7 \times 10^6$  BMDCs was 5.38 (1.49)%, and it was not significantly different from the percentage of GFP positive hepatocytes observed in GFP negative livers infused with the same quantity of cells (8.2 (1.9)%).

### Gunn rats

#### Analysis of blood and bile bilirubin concentrations

Thirty days after cell transplantation, the level of plasma unconjugated bilirubin was 35% lower than in basal conditions in rats receiving cell infusions (group K), while it was unchanged in control animals (group L) (fig 9). The decrease in plasma bilirubin was associated with the appearance of monoconjugated bilirubin in bile (0.9 (0.19)  $\mu$ mol/l;  $n = 6$ ), while no conjugates were detected in controls (fig 10). The alkaline methanolysis procedure adds specificity to the identification of bilirubin conjugates, as the trans-esterification reaction yielding bilirubin methylesters from bilirubin glucuronides takes place only in an alkaline environment. When plain methanol was substituted for alkaline methanol, the peak of bilirubin monomethylesters was selectively absent from the chromatogram. Such verification confirmed the presence of





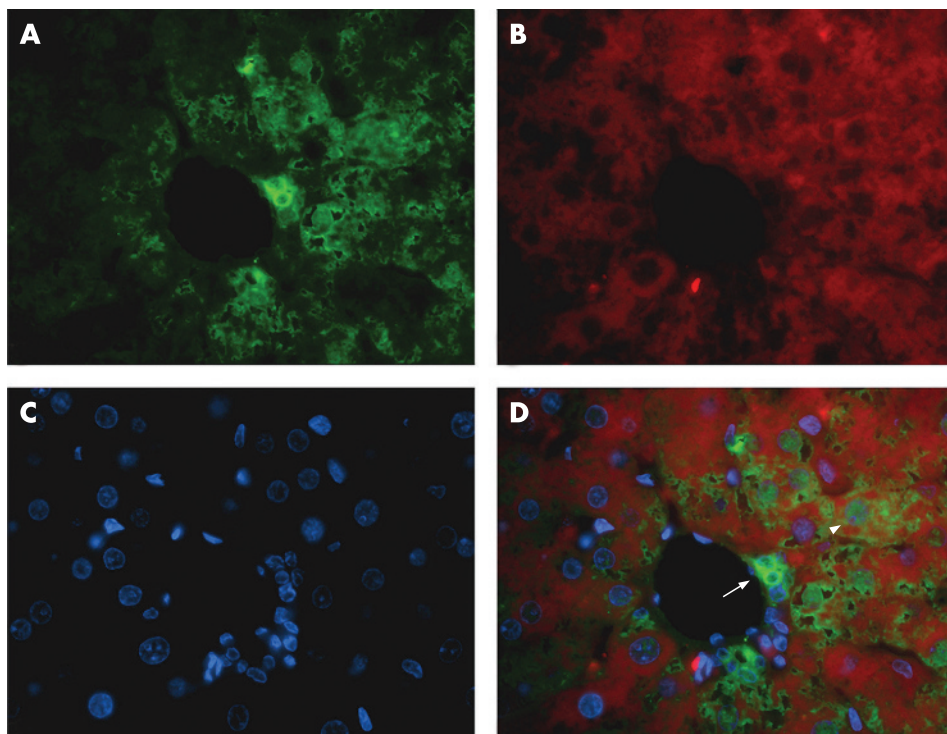
**Figure 2** Bone marrow derived cells (BMDCs) isolated from green fluorescent protein (GFP) positive rats acquire morphological features and phenotypic markers of differentiated hepatocytes after transplantation into liver injured Lewis recipients (group D). Serial sections of livers removed 72 hours after cell transplantation were stained for GFP (A) (C) and for CK-18 (B) (D). GFP positive cells are also positive for the epithelial marker CK-18. Several GFP positive cells with hepatocyte-like morphology are visible. The arrow indicates a binucleated cell, a feature typical of hepatocytes. Some GFP positive cells are smaller and have smaller nuclei (arrowheads) than the surrounding host hepatocytes. (Original magnification  $\times 40$ .) The two figures in the lower panel are: (E) a liver section from a Lewis rat (group E, GFP negative control) and (F) a liver section from a GFP-transgenic animal (GFP positive control), respectively. (Original magnification  $\times 10$ .)

bilirubin conjugates in the Gunn rats transplanted with BMDCs.

#### PCR analysis

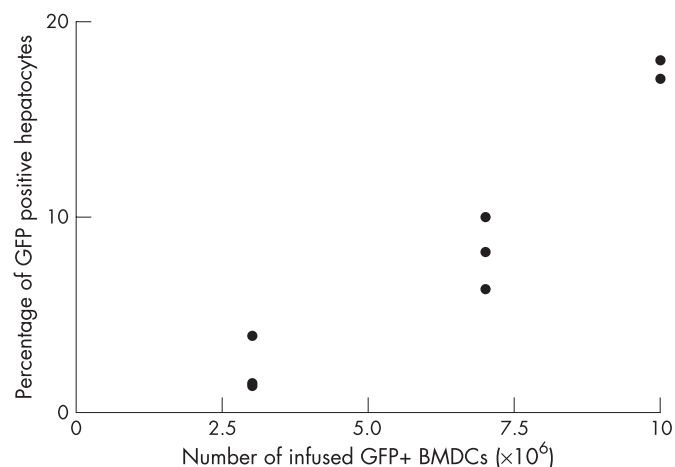
PCR analysis of uridine diphosphate glucuronosyltransferase (UDP-GTA) gene expression followed by digestion with the

restriction enzyme *MvaI* is illustrated in fig 11. The amplification product of the normal donor cDNA yields four bands (12, 67, 123, and 384 bp) after digestion. As the 12 bp band is lost during chromatography, only three bands are visible. The PCR product of Gunn rat cDNA lacks a restriction site on position 1271 for this enzyme, and therefore the digestion products yield



**Figure 3** Sections of livers removed 72 hours after cell transplantation (group D) were stained for GFP (A, fluorescent green) and  $\beta 2m$  (B, fluorescent red) combined with DAPI nuclear staining (C, fluorescent blue). Some smaller (presumably less differentiated) GFP positive cells stain negative for  $\beta 2m$  (arrow) while some larger (presumably more differentiated) GFP positive cells stain positive (arrowhead) (D).





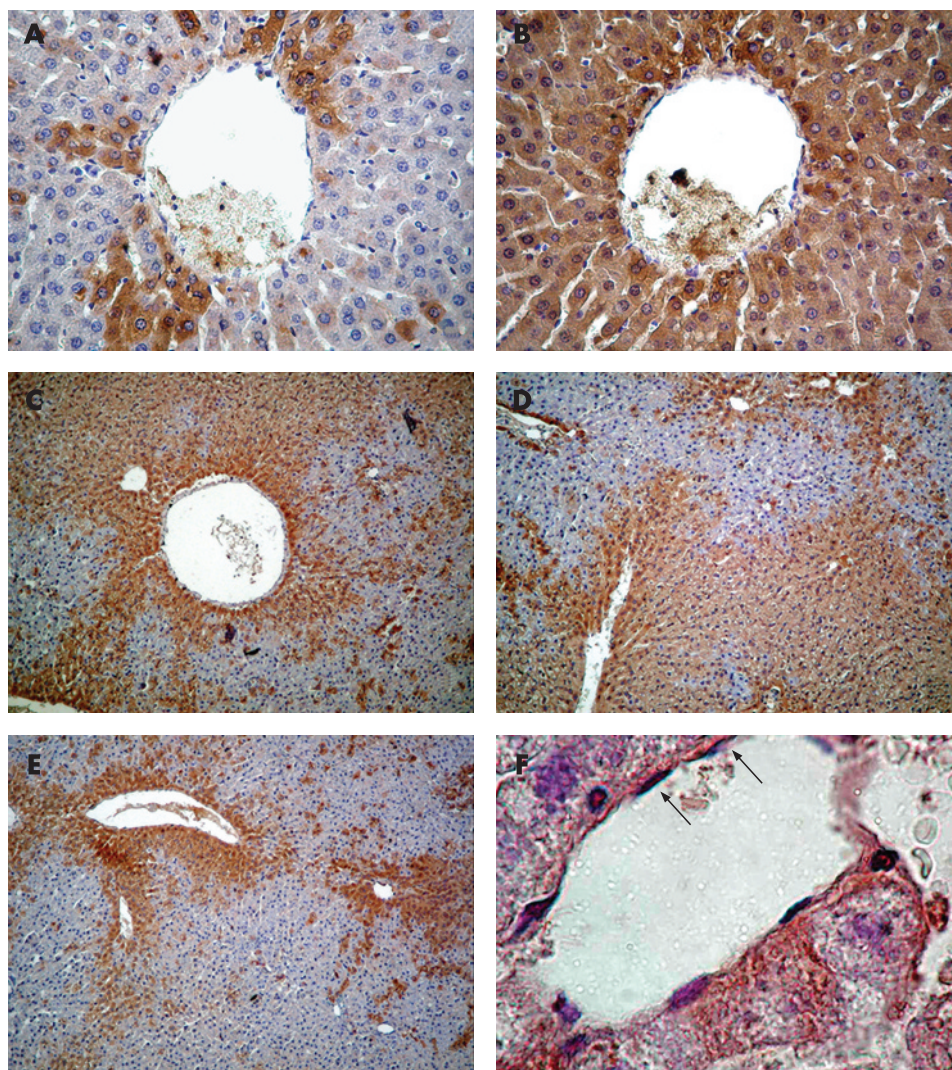
**Figure 4** Percentage of green fluorescent protein (GFP) positive hepatocytes (Hcs) one month after GFP positive bone marrow derived cell (BMDC) transplantation, as a function of the number of infused cells (group F;  $n=8$ ).

three bands (511, 67, and 12 bp, the last one not visible). In Gunn j/j rats, which received infusion of BMDCs from non-affected, wild type animals, four bands are visible, including those derived from digestion of the normal enzyme.

## DISCUSSION

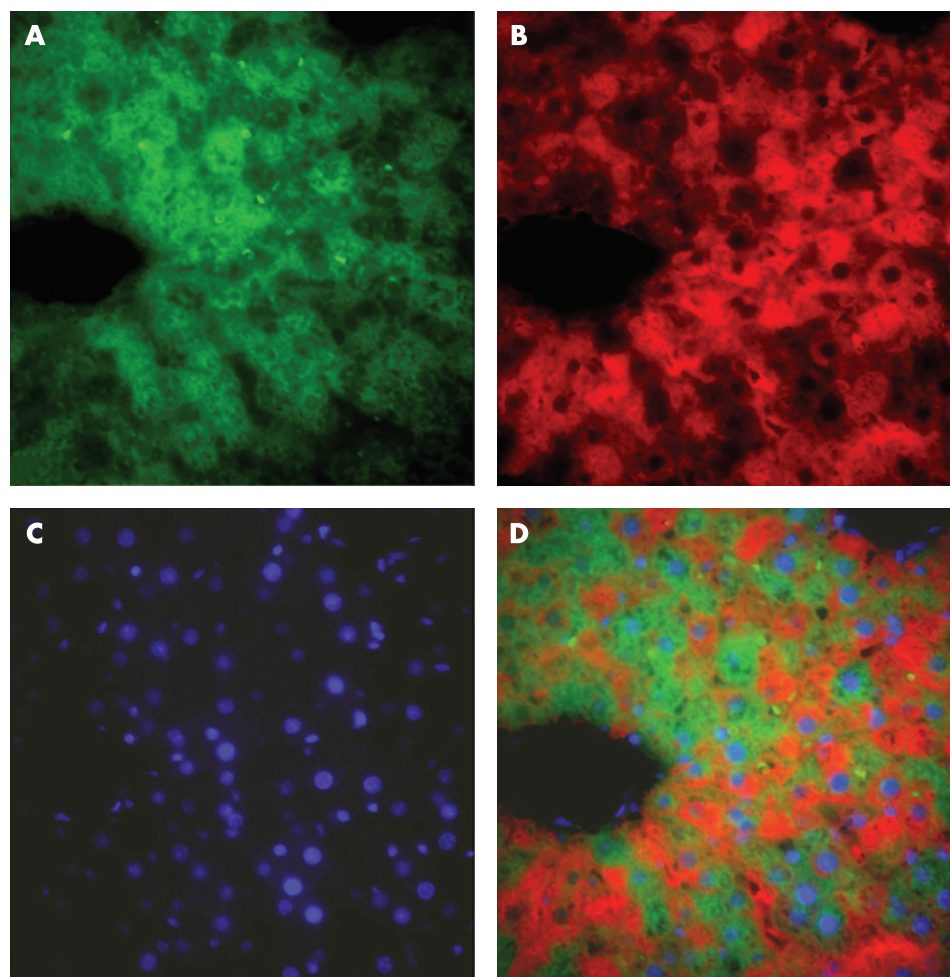
There has been much interest in cell therapy for liver metabolic disorders in the last decade, and several investigators have reported a significant clinical improvement following hepatocyte transplantation as an alternative to liver transplantation.<sup>9</sup> Major limitations are the limited availability of human hepatocytes and the limited long term efficacy. In theory, the use of BMDCs as an alternative to hepatocytes could solve both problems. The present work indicates first, that a significant parenchymal repopulation with hepatocyte-like cells derived from BMDCs can be induced even in metabolic disorders not associated with increased parenchymal cell turnover; second, that probably such repopulation is mostly due to differentiation rather than to cell fusion; and third, that such repopulation results in partial correction of a congenital hepatic metabolic disorder.

The extent of parenchymal repopulation with exogenous bone marrow derived, GFP positive hepatocyte-like cells averaged 0.4% in the liver lobes submitted to I/R 72 hours after infusion, but it increased by more than 20-fold after one month, suggesting proliferation of the exogenous cells. The histological finding showing large clusters of GFP positive hepatocytes supports this hypothesis. Continuous propagation of stem/progenitor cells in the absence of ongoing liver damage can be driven by cell-cell competition, with increased proliferation and reduced apoptosis of the transplanted cells when



**Figure 5** Bone marrow derived cells (BMDCs) isolated from green fluorescent protein (GFP) positive transgenic rats acquire morphological features and phenotypic markers of differentiated hepatocytes after transplantation into liver injured Lewis recipients (group F). Serial sections of livers removed four weeks after cell transplantation were stained for GFP (A) and for CK-18 (B). (C-E) Extensive repopulation of liver parenchyma with GFP positive hepatocyte-like cells four weeks after transplantation of  $10 \times 10^6$  GFP positive BMDCs (group F). Large clusters of GFP positive hepatocyte-like cells are mainly located in proximity of blood vessels. (F) GFP positive endothelial-like cells (arrows) are also visible in the ischaemic lobes. (Original magnification: (A), (B)  $\times 20$ ; (C), (D), (E)  $\times 10$ ; (F)  $\times 40$ .)

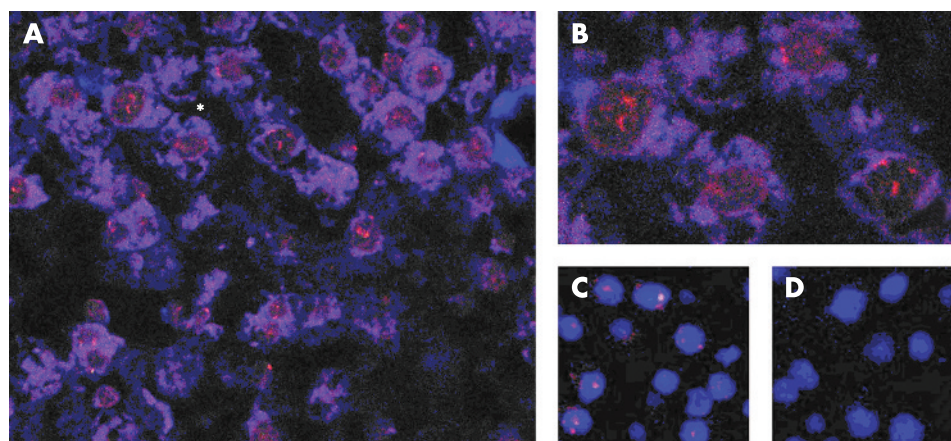




**Figure 6** Sections of livers removed one month after cell transplantation (group F) were stained for green fluorescent protein (GFP) (A, fluorescent green) and albumin (B, fluorescent red) combined with DAPI nuclear staining (C, fluorescent blue). GFP positive (bone marrow derived) hepatocyte-like cells stain more weakly or negatively for albumin when compared with the surrounding autologous (GFP negative) hepatocytes (D).

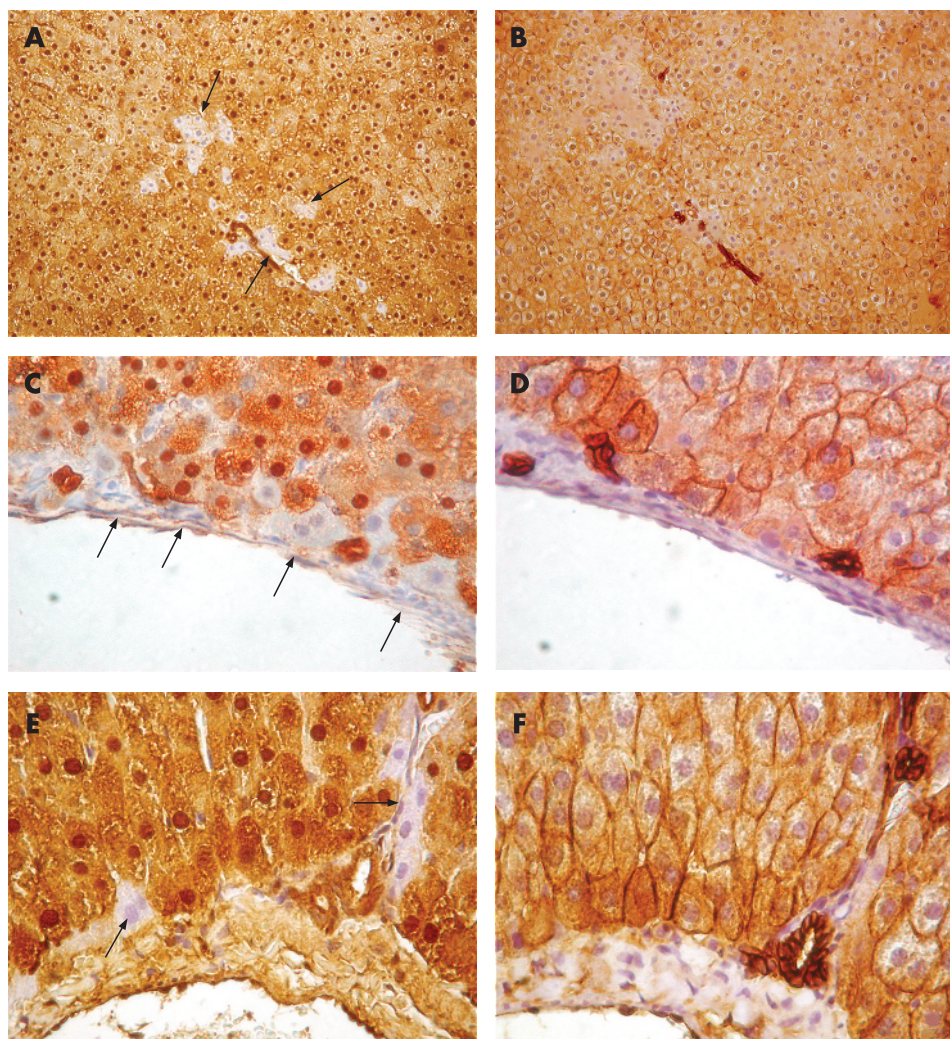
compared with host hepatocytes.<sup>10</sup> Previous work reported low or insignificant repopulation in several animal models of liver injury,<sup>3–11</sup> with the notable exception of a study by Jang *et al*,<sup>12</sup> which demonstrated 7.6% repopulation 48 hours after cell infusion. Possible explanations for such differences include the

selected stem cell population, the model of liver damage (also involving the endothelium), direct intraportal infusion *vs* systemic administration, and the survival advantage of transplanted cells in a milieu of endogenous cells injured by the previous I/R injury.



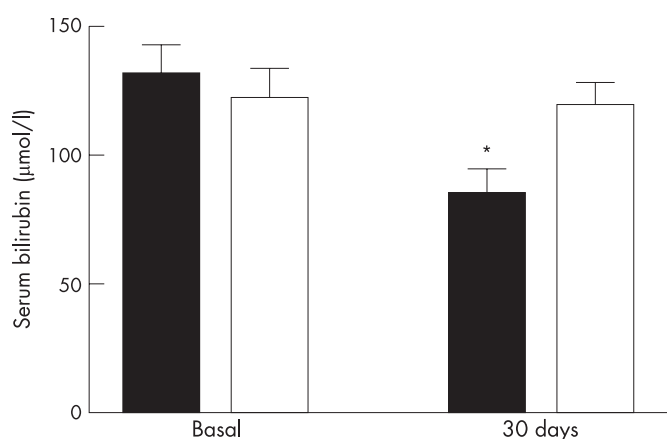
**Figure 7** Confocal microscopy analysis of liver tissue of a female rat one month after infusion of bone marrow derived cells (BMDCs) isolated from male GFP-transgenic rats (A) (B). Tissue has been processed with fluorescent in situ hybridisation (FISH) for the Y chromosome (Cy3, red nuclear signals) and immunostained for GFP (Cy5 labelling, blue cytoplasmic stain). Nuclei are dark as there is no nuclear DAPI labelling. Clusters of GFP positive hepatocytes incorporating the Y chromosome signal are visible throughout the parenchyma (60 $\times$ ); interestingly there are frequently multiple Y signals in a nucleus, denoting polyploidisation, a typical hepatocyte feature. (B) An enlarged section of the area indicated by an asterisk (\*) in the panel (A). (C, D) Fluorescent microscopy of control liver tissue: here DAPI (blue) is used to label the nuclei as no cytoplasmic fluorochrome is used; again Y chromosome detection is with the Cy3 labelled chromosome paint (red). (C) Male control liver shows nuclear Y chromosome labelling (red signals). (D) Female control livers have no nuclear Y chromosome labelling.





**Figure 8** Immunohistochemistry (IHC) on serial sections for green fluorescent protein (GFP) (A) (C) (E) and for CK-18 (B) (D) (F) one month after infusion of GFP negative bone marrow derived cells (BMSC) into GFP positive transgenic rats (group H). Animals were subjected to hepatic ischaemia/reperfusion (I/R) injury before cell infusion. Several GFP negative (arrows), CK-18 positive hepatocytes are visible within the liver parenchyma, suggesting differentiation from GFP negative BMDCs into hepatocyte-like cells rather than cell fusion between BMDCs and host hepatocytes. (Original magnification: (A), (B)  $\times 20$ ; (C), (D), (E), (F)  $\times 40$ .)

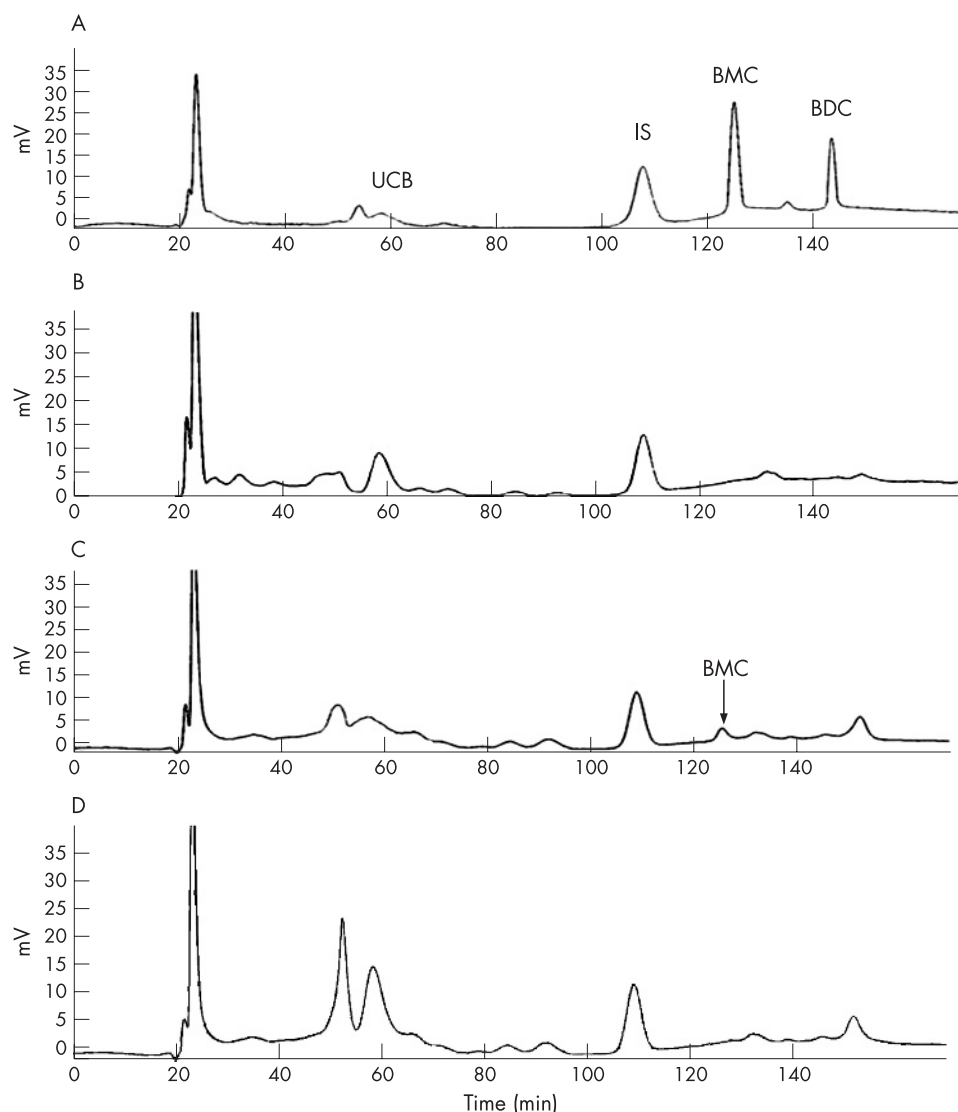
The infused BMDC population<sup>7 13</sup> was selected on the basis of negativity for  $\beta_2m$  and of positivity for Thy-1, a haematopoietic stem cell marker shared by hepatic oval cells.  $\beta_2m$  participates as an integral part in molecules of the major histocompatibility complex (MHC I).<sup>14</sup> Indeed, BMDCs share MHC I negativity.<sup>15</sup>



**Figure 9** Serum bilirubin immediately before and 30 days after bone marrow derived cell (BMDC) transplantation in Gunn rats. Black bars represent animals transplanted with  $10 \times 10^6$  BMDCs from wild type non-jaundiced rats. Controls (white bars) received vehicle only. Data are expressed as means, error bars = standard deviation, n=6. \* Different from basal conditions, p<0.05.

Fetal hepatoblasts<sup>16</sup> as well as neuronal stem cells<sup>17</sup> are negative for MHC class I antigens, suggesting that such a feature could be a general marker of stemness/immaturity. A subpopulation of  $\beta_2m$  negative BMDCs was shown to differentiate into cardiomyocytes and to reconstitute the myocardium in a model of myocardial infarction.<sup>18</sup> Interestingly,  $\beta_2m$  negative cells appeared to be selected (or  $\beta_2m$  downregulated) following transplantation of human cord blood stem cells in the liver of SCID mice.<sup>19</sup> More recently, a population of  $\beta_2m$  negative, c-Met positive cells was shown to differentiate into hepatocyte-like cells in culture.<sup>20</sup> That  $\beta_2m$  negative cells are the true population responsible for the liver parenchymal repopulation in the present work is suggested by the presence of  $\beta_2m$  negative cells integrating in liver parenchyma and showing morphological signs of hepatocytic differentiation 72 hours after infusion (fig 1).

I/R is a powerful stimulus for stem cell homing and repair of the injured tissue, probably mediated through the rapid induction of the stem cell factor in various tissues, including the myocardium,<sup>4</sup> the retina,<sup>21</sup> and the liver.<sup>22</sup> Moreover, macrophage activation secondary to the ischaemic damage is associated with the release of cytokines, activating endothelial cells with release of adhesion molecules, facilitating the anchorage and translocation of circulating stem cells.<sup>23 24</sup> Ischaemic damage is also associated with the induction of SDF-1, a key molecule in stem cell recruitment to injured organs and tissues.<sup>25</sup> In addition, the endothelial damage associated with I/R injury might facilitate translocation and



**Figure 10** Alkaline methanolysis-HPLC analysis of bilirubin pigments in bile. (A) Bile from a wild type non-jaundiced rat. (B) Bile from a *j/j* (jaundiced) Gunn rat. (C) Bile from a *j/j* Gunn rat one month after transplantation with bone marrow derived cells (BMDCs) from wild type animals. (D) When plain methanol was substituted for alkaline methanol in the above bile sample, the peak of bilirubin monomethylesters was selectively absent from the chromatogram, confirming its derivation from bilirubin monoglucuronide (see Results). BDC, bilirubin diconjugate; BMC, bilirubin monoconjugate; HPLC, high performance liquid chromatography; IS, internal standard; UCB, unconjugated bilirubin.

parenchymal engraftment of infused cells.<sup>26</sup> Indeed, BMDCs were invariably observed only within the blood vessels 72 hours after infusion into normal livers, while engraftment of  $\beta_2$ m negative cells was evident at the same time in I/R injured livers (figs 1 and 3).

In pilot experiments, we did not observe significant homing of fluorescent labelled BMDCs when the cells were administered immediately after releasing the vascular clamps. Thus the activation of biochemical signals was necessary in order to induce homing and engraftment of BMDCs.

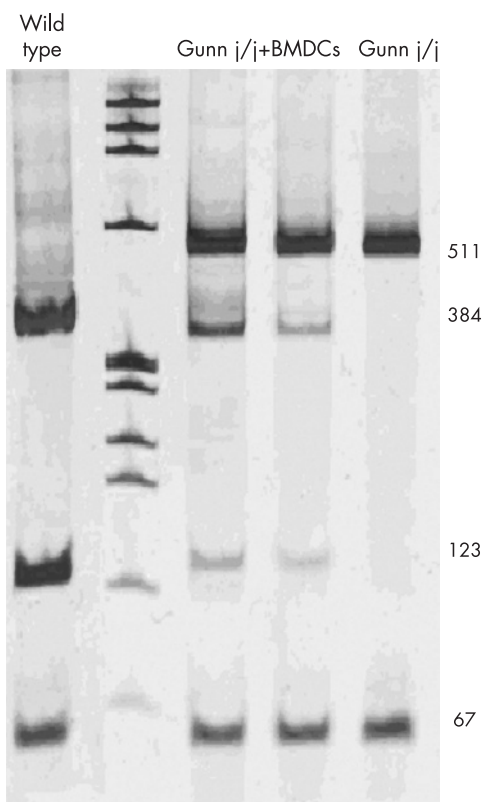
Previous studies demonstrating insignificant liver parenchymal repopulation with BMDCs involved either bone marrow transplantation with subsequent random migration to the liver of an unknown cell population<sup>3,9</sup> or the intravenous systemic delivery of a selected cell phenotype.<sup>27</sup> As the behaviour of stem cells is strongly determined by their niche,<sup>28</sup> direct intraportal infusion of BMDCs might have contributed to the relatively high hepatic repopulation with bone marrow derived hepatocytes observed in the present study. The importance of the local concentration of transplanted cells is also supported by the finding of a positive relation between the number of administered cells and the extent of parenchymal repopulation (fig 4).

Studies with hepatocyte transplantation have shown that a metabolic liver deficiency can be corrected by transplanting as little as 2% of the total hepatocyte mass, 25–50% of which will

probably engraft.<sup>24</sup> According to our results in group F, transplantation of  $10 \times 10^6$  BMDCs results in 20% repopulation of the infused lobes, corresponding to about 30% of the total liver mass. Therefore the magnitude of the repopulation relative to the whole liver is about 6–7%. Such parenchymal repopulation with hepatocytes derived from healthy BMDCs was able to partially correct a metabolic liver disease, as demonstrated by the decrease in serum bilirubin associated with the appearance of conjugated bilirubin in the bile of Gunn rats. The metabolic improvement was relatively small when compared with previous reports on hepatocyte transplantation, where a similar repopulation rate with exogenous wild type hepatocytes in Gunn rats resulted in up to a 50% decrease in blood bilirubin.<sup>29,30</sup> Clinical hepatocyte transplantation followed by phenobarbitone (phenobarbital) treatment in a girl with Crigler–Najjar disease produced a 32% decrease in serum bilirubin.<sup>31</sup> Collectively, these results confirm that even the appearance of a low metabolic activity can produce dramatic changes in serum bilirubin levels.

Our results do not exclude a fusion process<sup>2,32,33</sup> as a possible mechanism partially contributing to the observed repopulation of liver parenchyma with “normal” hepatocytes. However, the extent of liver parenchymal repopulation with exogenous cells was similar in the GFP positive to GFP negative and in the GFP negative to GFP positive groups. Moreover, fusion was





**Figure 11** Polymerase chain reaction (PCR) analysis of the UDP-GTA gene following digestion with the restriction enzyme *MavI*. Acrylamide gel electrophoresis of the amplification product of the cDNA from wild type and Gunn *j/j* rats yields different patterns after digestion with the restriction enzyme. In Gunn *j/j* rats, which received infusion of bone marrow derived cells (BMDCs) from non-affected, wild type animals, four bands are visible, including those derived from digestion of the normal enzyme (384 and 123 bp). See text for additional explanations.

described as a late phenomenon,<sup>2 32 33</sup> while in the present study, evidence of hepatocytic differentiation of BMDCs was observed already 72 hours after infusion (figs 1 and 2), as in the observations reported by Jang *et al* in liver injured mice.<sup>12</sup> More recent work further supports the hypothesis that, through subjection to local growth factors and cell-cell contact, the BMDCs may undergo changes in cell fate, mimicking the phenotype of the surrounding cells in the absence of cell fusion.<sup>34</sup> Interestingly, the BMDCs had high levels of CK-18 expression, similar to oval cells.<sup>35</sup> However, the expression of albumin was lower than in the surrounding indigenous non-bone-marrow derived hepatocytes (fig 6), suggesting that these hepatocyte-like cells were less metabolically competent, in keeping with a lower than expected metabolic effect in the Gunn rat.

A limitation of the present study is the short observation period following the metabolic improvement in treated animals. Even if exogenously derived cells showed normal appearance at histology one month after transplantation of BMDCs, we cannot exclude the possibility that longer observation times would result in improved expression of metabolic activity. Another important issue is the life span of transplanted cells, as loss of the metabolic effect over time has been observed in clinical hepatocyte transplantation.<sup>3</sup> Clearly, much longer observation times would be required to answer these questions.

## Conclusions

We achieved partial correction of a genetic liver metabolic disorder in a rat model by reversible I/R injury followed by

transplantation of BMDCs, which differentiated into hepatocyte-like cells and showed specific (although lower than expected) metabolic activity. These results provide preliminary proof-of-principle that such a procedure could be used as a therapeutic tool in patients with inherited disorders of liver metabolism.

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